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# The Cortical Peptidoglycan from Spores of *Bacillus megaterium* and *Bacillus subtilis* Is Not Highly Cross-Linked

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**Determination by amino acid analyses of the percentage of diaminopimelic acid in the spore cortex of *Bacillus megaterium* and *Bacillus subtilis* which is involved in interpeptide cross-links gave values of 31 to 37%. This finding supports the idea that the cortex volume could undergo significant changes in response to changes in pH or ionic strength and could thus play an active role in reducing the water content of the spore protoplast during sporulation.**

In the bacterial endospore, the metabolically inactive protoplast is encased in multiple integuments. The most prominent of these are the spore coats, composed primarily of protein, underlying which is the spore cortex, composed primarily of peptidoglycan. In contrast to the spore coats, the spore cortex appears to play a major role in spore heat resistance. Spores which contain defective coat layers or which have been chemically stripped of their coats maintain full, or nearly full, heat resistance (4). However, changes in the structure or volume of the spore cortex result in a corresponding loss of heat resistance (1, 5, 10). The involvement of the cortex in spore heat resistance has generally been attributed to its role in maintaining the dehydrated state of the spore protoplast (4). In addition, models which predict a role for the cortex in bringing about the dehydration of the protoplast, in addition to maintaining it, have been presented. In the contractile cortex (6) and the anisotropically expansive cortex (13) models, changes in the diameter or volume of the cortex would effectively squeeze water from the spore core. These models are compatible with the classical model of spore cortex structure, in which there is a low to moderate degree of cross-linking of the peptidoglycan side chains (15), a characteristic that allows significant shrinkage and swelling of the cortex in response to changes in pH or ionic strength (4, 9).

In an early characterization of spore cortex structure, Warth and Strominger found that 35% of the cortical muramic acid residues were substituted with peptide side chains (15). However, only 19% of the ε-amino groups of the diaminopimelic acid (Dpm) residues in these side chains were involved in interpeptide cross-links, with the remainder having a free ε-amino group. These values were determined by using cortex solubilized by lysozyme digestion of spores which had first been heat inactivated and mechanically disrupted.

More recently, Marquis and Bender (7) reported that cortex isolated from spores by a chemical extraction procedure contained very few free amino groups, suggesting a highly cross-linked structure. Their data further suggested that mechanical shearing significantly increased the number of free amino groups found in the cortex. They theorized that the native spore cortex possesses a rigid, highly cross-linked structure that serves only to maintain pressure on the spore core and that the loosely cross-linked structure originally proposed was simply an artifact of the cortex purification method used at that time.

Given the disparity between the degrees of cross-linking of the spore cortex in the published reports, and the significance of this value in terms of models of spore cortical function, we have attempted to repeat and extend the analyses of Marquis and Bender. Thus, we carried out analyses only on chemically extracted spores. However, our analyses of the degree of cortical peptide cross-linking relied not on measurement of free amino groups but rather on the amount of Dpm present in acid hydrolysates before or after the reaction of free amino groups with 1-fluoro-2,4-dinitrobenzene (FDNB). In our initial work, we used the cortex purification scheme of Marquis and Bender (7) with spores of *Bacillus subtilis* 168 prepared as described previously (8). Amino acid analysis of the resulting cortical sacculi (7) demonstrated that the preparation contained almost twice as much protein as peptidoglycan (Table 1), and electron microscopic examination revealed that significant amounts of spore coat and possibly spore core proteins remained in the preparation (data not shown). Similar results were obtained with spores of *Bacillus megaterium* QMB1551 (data not shown). The difference between the amounts of protein detected in our cortical sacculi preparation and in that of Marquis and Bender (7) may be due to species or strain differences affecting the efficiency of spore coat removal or to our more rigorous method of protein measurement. When we attempted to determine the number of free amino groups present in our cortical sacculi by direct reaction with FDNB, according to the protocol of Marquis and Bender (7), we obtained values over twice the predicted amount of total Dpm (data not shown). We attribute the majority of these free amino groups to the unextracted protein, not to Dpm.

In an attempt to obtain more highly purified spore cortex preparations, we subjected intact spores to a number of additional extraction steps (Table 1). The final method we used involved decoating of the spores twice in a solution containing 50 mM Tris-HCl (pH 8.0), 8 M urea, 1% (wt/vol) sodium dodecyl sulfate (SDS), and 50 mM dithiothreitol at 37°C for 1 h, followed by three washes in distilled water. These and all following steps were carried out with ≤5 mg of dry spores per ml. The decoated spores were then suspended in 5% trichloroacetic acid (TCA), heated to 90°C for 6 min,

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TABLE 1. Levels of protein, cortex, and Dpm in *B. subtilis* spores after various extraction procedures[a]

| Spore extraction step[b] | Amt (μg) of: | | | Dpm recovery (%) |
|---|---|---|---|---|
| | Dpm | Cortex[c] | Protein[d] | |
| None | 11.9 | 128 | 336 | 100 |
| Decoating | 12.6 | 135 | 318 | 106 |
| TCA[e] | 9.6 | 103 | 230 | 81 |
| SDS-TCA-SDS | 7.7 | 83 | 114 | 65 |
| Muramidase supernatant | 6.8 | 73 | 19 | 57 |
| Muramidase pellet | 0.2 | 2 | 90 | 2 |

[a] Spores were subjected to sequential extraction treatments, and samples were hydrolyzed and amino acids were analyzed as described in the text. Most of the losses in spore cortex and Dpm in the TCA and SDS extractions were due to physical losses of spores during the multiple centrifugation steps needed for these procedures.

[b] Extractions were carried out sequentially with one preparation of spores.

[c] Calculated from the amount of Dpm, on the basis of molar ratios of *N*-acetylglucosamine *N*-acetylmuramic acid alanine glutamate Dpm of 2.91: 2.91:2.5:1:1 (15).

[d] Total amino acids detected, minus the alanine, glutamate, and Dpm contributed by the cortex.

[e] The cortical sacculi analyzed by Marquis and Bender (7) were routinely brought through this extraction step and, in some cases, further extracted once with SDS.

centrifuged, and washed in 0.5 M Tris-HCl (pH 9.5). The extracted spores were then boiled in a solution containing 50 mM Tris-HCl (pH 8.0), 3% (wt/vol) SDS, and 50 mM dithiothreitol for 20 min and then washed three times in distilled water. The hot TCA and SDS-dithiothreitol treatments were then repeated. In some experiments, spore cortex peptidoglycan was solubilized by digestion with muramidase (Sigma; mutanolysin, 60 U/mg of spores) in 0.1 M $NH_4HCO_3$ for 16 h at 37°C. Samples from various steps of the purification were acid hydrolyzed (6 N HCl, 6 h, 100°C) before or after treatment with FDNB as described below; FDNB-treated spores were washed with water prior to acid hydrolysis. Amino acid analyses were carried out on acid hydrolysates with a Beckman System 7300 amino acid analyzer (2), with the elution positions of glucosamine, muramic acid, and Dpm determined separately by using purified standards. Control experiments showed that harsher acid hydrolysis (6 N HCl, 1 h, 150°C) yielded no more (<10%) total amino acid or Dpm. The results from these analyses indicated that coat-stripped *B. subtilis* spores extracted twice with hot TCA and SDS retained a significant amount of protein (Table 1). Similar results were obtained with *B. megaterium* spores (data not shown). Electron microscopy of *B. subtilis* spores after these extractions suggested that significant material remains in the spore coat, cortex, and core regions (Fig. 1a). Treatment of the extracted spores with muramidase resulted in solubilization of >95% of the Dpm, but it solubilized only a small amount of protein (Table 1). The resulting spores had lost the spore cortex layer, but they retained the material in the coat and core regions (Fig. 1b).

In order to determine the percentage of the cortical Dpm which was cross-linked, we treated the extracted spores with 0.2% FDNB for 16 h at room temperature in 80 mM $NaHCO_3$ (pH 8.0) and 20% ethanol prior to acid hydrolysis and amino acid analysis. Control experiments showed that the FDNB treatment modified >95% of the lysine in a model cortical peptide, L-Ala-D-Glu-L-Lys-D-Ala, but modified only 33% of the lysine and 30% of the Dpm in decoated spores (data not shown). We felt that this incomplete modification would not produce a valid estimation of cross-linking. A single TCA extraction, however, rendered >80% of the lysine sensitive to modification, whereas further TCA and SDS extractions had little effect (<10%) on the extent of lysine and Dpm reaction with FDNB (data not shown). This suggests that neither of these extraction procedures is producing appreciable hydrolysis of cross-links. Within each experiment, we found that the ratios of a number of amino acids (including Ala, Leu, Ile, Val, and Met) did not vary between control and FDNB-treated samples. Therefore, the amount of Dpm in these samples was expressed as a ratio to the amount of valine, an amino acid that was well resolved in our chromatograms. The amount of Dpm detected after

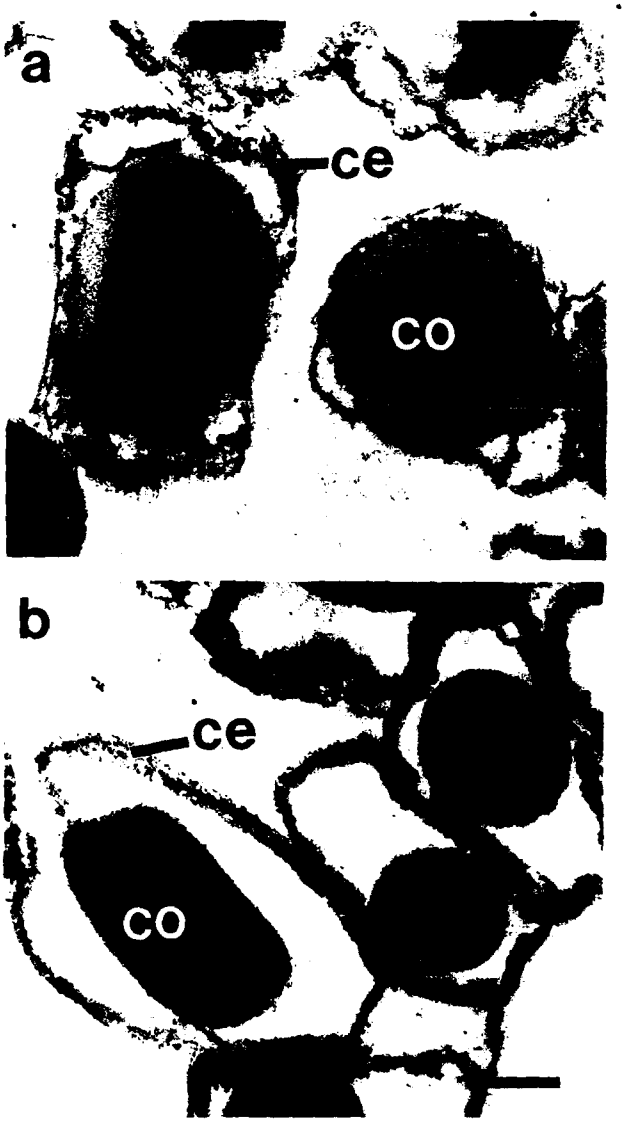


FIG. 1. Electron micrographs of *B. subtilis* spores after various extraction procedures. (a) Spores of *B. subtilis* were taken through the SDS-TCA-SDS extraction step as shown in Table 1, and then samples were fixed, postfixed, stained, and photographed as described previously (11). (b) Spores of *B. subtilis* were taken through the muramidase step as shown in Table 1 and treated and photographed as described above. Abbreviations: ce, spore coat and exosporium region; cx, spore cortex; co, spore core. Bar, 0.18 μm.

TABLE 2. Percentage of cortical Dpm cross-linked in *B. subtilis* spores and cells and in *B. megaterium* spores[a]

| Sample | FDNB treatment | Amt of Dpm amt of Val[b] | % Cross-linked Dpm[c] |
|---|---|---|---|
| *B. subtilis* spores | − | 6.41[d] | |
| | + | 2.77[d] | 37 |
| *B. megaterium* spores | − | 2.83 | |
| | + | 0.91 | 31 |
| *B. subtilis* vegetative cells | − | 6.08 | |
| | + | 2.71 | 42 |

[a] The spores to be analyzed were taken through the SDS-TCA-SDS extraction step shown in Table 1. Vegetative cells were treated similarly but with the decoating step omitted. Samples were then acid hydrolyzed before or after FDNB treatment, and amino acids were determined as described in the text.

[b] Similar relative ratios were achieved with leucine and alanine.

[c] Calculated as described in the text. The value for *B. subtilis* spores is the mean of four determinations, with a standard deviation of 5.

[d] Mean values for four determinations, including one from each of three spore preparations. The ratios varied with the degree of protein removal during extraction treatments.

FDNB treatment indicates the amount of Dpm that was cross-linked and thus did not have a free amino group to react with FDNB. The cross-linkage values we report are corrected for the efficiency of FDNB reaction as defined by the percentage of lysine modified in each sample, which varied from 84 to 98%. These analyses demonstrate that the Dpm in *B. megaterium* and *B. subtilis* spore cortex was 31 and 37% cross-linked, respectively (Table 2). We also examined the cross-linking of Dpm in *B. subtilis* vegetative cell walls, purified by our method (omitting the decoating steps), and found that the Dpm was 42% cross-linked. This is in good agreement with previously reported values of 41% Dpm cross-linking for vegetative cell wall peptidoglycan purified by a different protocol (14) and 33% Dpm cross-linking for vegetative peptidoglycan produced in the presence of the antibiotic cephalothin and analyzed by nuclear magnetic resonance and gas chromatography-mass spectrometry (3).

Our results for spore cortex are very similar to those of Warth and Strominger (15). We found that Dpm is present at approximately 25% of the molar quantity of glucosamine in *B. subtilis* spore cortex peptidoglycan (not shown) and that only 37% of this Dpm is involved in peptide cross-links. The difference between our value (37%) and that previously reported by Warth and Strominger (19%) (15) could be due to a difference in the cortex purification methods or to a strain difference. Both values are, however, extremely different from those derived from the data of Marquis and Bender, which indicate that 67 to 96% and >90% of the cortical Dpm is cross-linked in *B. megaterium* and *Bacillus cereus* spores, respectively (7). Unfortunately, despite several attempts and variations of our protocol, we could not reproduce the results of Marquis and Bender. The finding of a low degree of interpeptide cross-linking in spore cortex prepared by two different methods leads us to maintain that the cortex may indeed be capable of mechanical action in response to changes in pH or ionic strength; however, volume measurements of more highly purified cortex will be required to verify this. The spore cortex may thus have a role in attaining as well as maintaining the dehydration of the spore core, a dehydration which may cause both spore heat resistance and spore dormancy (4, 12).

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